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Isolation of Shiga Toxin–Producing *Escherichia coli* Serogroups O26, O45, O103, O111, O121, and O145 from Ground Beef Using Modified Rainbow Agar and Post–Immunomagnetic Separation Acid Treatment†

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ABSTRACT

It is estimated that at least 70% of human illnesses due to non-O157 Shiga toxin–producing *Escherichia coli* (STEC) in the United States are caused by strains from the top six serogroups (O26, O45, O103, O111, O121, and O145). Procedures for isolating STEC from food products often use plating media that include antimicrobial supplements at concentrations that inhibit background microflora growth but can also inhibit target STEC growth. In this study, an agar medium with lower supplement concentrations, modified Rainbow agar (mRBA), was evaluated for recovery of STEC serogroups O26, O45, O103, O111, O121, and O145 from ground beef enrichments. A post–immunomagnetic separation (IMS) acid treatment step was additionally used to reduce background microflora and increase recovery of target STEC strains. Ground beef samples (325 g) were artificially contaminated with STEC and confounding organisms and enriched for 15 h. Recovery of the target STEC was attempted on the enrichments using IMS and plating onto mRBA and Rainbow agar (RBA). Additionally, acid treatment was performed on the post-IMS eluate followed by plating onto mRBA. Using the combination of mRBA and acid treatment, target STEC were isolated from 103 (85.8%) of 120 of the low-inoculated samples (1 to 5 CFU/325-g sample) compared with 68 (56.7%) of 120 using no acid treatment and plating onto RBA with higher levels of novobiocin and potassium tellurite. The combination of acid treatment and mRBA provides a significant improvement over the use of RBA for isolation of STEC serogroups O26, O45, O103, O111, O121, and O145 from raw ground beef.

It has been estimated that at least 70% of human illnesses associated with non-O157 Shiga toxin–producing *Escherichia coli* (STEC) in the United States are from one of the top six STEC serogroups, O26, O45, O103, O111, O121, and O145 *(6)*. The U.S. Department of Agriculture, Food Safety and Inspection Service (USDA FSIS) recently declared the top six STEC serogroups to be adulterants in raw beef *(1)*. Current methods for isolation of non-O157 STEC include the use of tellurite cefixime–sorbitol MacConkey agar (TC-SMAC), Levine's eosin methylene blue (L-EMB), and Rainbow agar O157 (RBA) *(9, 11)*. Potassium tellurite has been used as an antimicrobial supplement in plating media to select for *E. coli*, mainly strains of O157:H7 *(19)*. However, it has been reported that strains from the top six non-O157 STEC serogroups, mainly from serogroup O103, is inhibited by the concentration of potassium tellurite used in both TC-SMAC agar (2.5 mg/liter) and RBA (0.8 mg/liter) *(12)*. In light of the potential selection bias against tellurite-sensitive STEC strains, there is a need for formulating an alternative plating medium that supports growth of non-O157 STEC yet allows selectivity and differentiation from non–*E. coli* and other *E. coli* strains. Preliminary work in our laboratory demonstrated that modified Rainbow agar (mRBA) with 0.05 mg/liter cefixime, 0.15 mg/liter potassium tellurite, and 5 mg/liter novobiocin was able to support growth of more STEC strains from our collection than the formulation for RBA (0.8 mg/liter potassium tellurite and 10 mg/liter sodium novobiocin) as currently described for isolation of *E. coli* O157:H7 in the USDA FSIS *Microbiology Laboratory Guidebook* (MLG) *(3)*.

High background flora encountered in complex matrices, such as ground beef, can confound recovery of STEC on currently available selective and differential plating media. Often, as is the case with STEC, there is a difficulty in identifying a single phenotypic characteristic that can be used to selectively grow and isolate the target. The use of immunomagnetic separation (IMS) using antibody-coated

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† Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

TABLE 1. *Non-O157 STEC strains and confounding organisms used in experiments demonstrating recovery from inoculated ground beef*[a]

Organism	Laboratory identifier	Source	Toxin type
Escherichia coli O26:H11	LIMS#100027798	ARS ERRC	stx_1
E. coli O26:H11	LIMS#100032474	ARS ERRC	stx_1
E. coli O26:H11	LIMS#100032475	ARS ERRC	stx_1, stx_2
E. coli O26:H2	LIMS#201033446	ARS ERRC	stx_1
E. coli O45:H2	LIMS#100032476	ARS ERRC	stx_1
E. coli O45:H2	LIMS#100032916	ARS ERRC	stx_1
E. coli O45:NM	LIMS#100036526	MSU	stx_1
E. coli O45:H2	LIMS#201033447	ARS ERRC	stx_1
E. coli O103:H2	LIMS#100027795	ARS ERRC	stx_1
E. coli O103:H25	LIMS#100032478	ARS ERRC	stx_1
E. coli O103:H11	LIMS#100032479	ARS ERRC	stx_1, stx_2
E. coli O103:H2	LIMS#201033448	ARS ERRC	stx_1
E. coli O111:NM	LIMS#100032480	ARS ERRC	stx_1, stx_2
E. coli O111:[H8]	LIMS#100027761	ARS ERRC	stx_1, stx_2
E. coli O111:H8	LIMS#100036525	MSU	stx_1, stx_2
E. coli O111:H$^-$	LIMS#201033449	ARS ERRC	stx_2
E. coli O121:H19	LIMS#100032481	ARS ERRC	stx_1, stx_2
E. coli O121	LIMS#100018370	ARS WRRC	stx_1
E. coli O121	LIMS#100036523	MSU	stx_2
E. coli O121:H19	LIMS#201033450	ARS ERRC	stx_2
E. coli O145:NM	LIMS#100032482	ARS ERRC	stx_1,stx_2
E. coli O145:H18	LIMS#201013659	ARS ERRC	stx_2
E. coli O145:[H28]	LIMS#100036530	MSU	stx_1
E. coli O145:H$^-$	LIMS#201033451	ARS ERRC	stx_1, stx_2
Morganella morganii	LIMS#201033452	FSIS OSEL	NA
Enterobacter cloaceae	LIMS#201033453	FSIS OSEL	NA
Citrobacter freundii	LIMS#201033454	FSIS OSEL	NA
Enterobacter sakazakii	LIMS#201033455	FSIS OSEL	NA
Enterobacter cloacae	LIMS#201033456	FSIS OSEL	NA
Klebsiella pneumoniae	LIMS#201033457	FSIS OSEL	NA

[a] ARS ERRC, USDA Agriculture Research Service, Eastern Regional Research Center, Wyndmoor, PA; ARS WRRC, USDA Agriculture Research Service, Western Regional Research Center, Albany, CA; MSU, Michigan State University STEC Center, East Lansing, MI; FSIS OSEL, USDA FSIS Outbreaks Section of Eastern Laboratory, Athens, GA; NA, not applicable.

paramagnetic beads has been reported to increase the sensitivity and selectivity of pathogen isolation procedures *(8, 17, 18)*. Acid treatment at pH 2 has been demonstrated to be effective in reducing background flora and enhancing target STEC recovery *(7, 12, 13)*. Acid tolerance and acid resistance is regarded as an important intrinsic property of *E. coli* strains, thus enabling the organism to survive acidic environments such as the stomach *(4, 5, 10)*. Commensal *E. coli* and pathogenic STEC strains have been reported to survive exposure to low pH levels (pH 2.0) for several hours, whereas common competitor organisms such as *Citrobacter freundii, Enterobacter aerogenes, Proteus mirabilis,* and *Klebsiella pneumoniae* have been demonstrated to be lethally injured at those pH levels *(12, 13)*. Combining the intrinsic acid tolerance of STEC and the specificity imparted through IMS potentially presents an effective method for isolation of STEC from food or environmental samples. The aim of this study was to evaluate the use of mRBA as a plating medium following IMS for isolation of STEC serogroups O26, O45, O103, O111, O121, and O145 from artificially contaminated ground beef enrichments. Additionally, a post-IMS acid treatment step intended to reduce background microflora was evaluated for its use in conjunction with plating onto mRBA. We demonstrate that plating onto mRBA alone or in conjunction with acid treatment can facilitate recovery of target STEC serogroups O26, O45, O103, O111, O121, and O145 from ground beef enrichments.

MATERIALS AND METHODS

Bacterial strains. Target STEC bacterial strains used for the recovery experiments are listed in Table 1. Strains were maintained on CryoCare beads (Key Scientific, Stamford, TX) and stored at −80°C until use. Beads were removed from vials and streaked for isolation onto tryptic soy agar (TSA) with 5% sheep blood (Remel, Lenexa, KS) and incubated at 37°C for 18 to 24 h. The confounding organisms (Table 1) used in acid sensitivity experiments and recovery experiments were isolated from ground beef enrichments following IMS with anti-O103 beads (Invitrogen, Carlsbad, CA) and subsequent plating onto RBA in preliminary experiments. The organisms were identified biochemically using GNI+ biochemical cards on the VITEK instrument (bioMérieux, Hazelwood, MO) and genetically using 16S sequencing.

Plating media preparation. RBA O157 (Biolog, Hayward, CA) was prepared as recommended by the manufacturer and supplemented with 0.8 mg/liter potassium tellurite (BD Biosciences, Franklin Lakes, NJ) and 10 mg/liter sodium novobiocin as described in the USDA FSIS MLG ''Appendix 1: Media and

Reagents'' *(3).* The modified RBA O157 (mRBA) formulation contained the following supplement concentrations: 0.15 mg/liter potassium tellurite, 5 mg/liter sodium novobiocin, and 0.05 mg/liter cefixime trihydrate (USP, Rockville, MD). Cefixime was added as an antimicrobial supplement to the plating medium due to its reported inhibition of *Proteus* spp. and other confounding organisms *(14, 15).* The agar bases of the plating media were dissolved in water, autoclaved as recommended by the manufacturer, and cooled to approximately 50°C prior to adding the appropriate antimicrobial supplements.

Acid treatment procedure using pure cultures. A representative isolate of each of the six non-O157 STEC serogroups of interest and each of six confounding organisms isolated from ground beef were grown overnight on TSA with 5% sheep blood at 37°C. Following incubation, a single colony from each strain was used to individually inoculate 3 ml of brain heart infusion (BHI) broth. The inoculated broths were incubated statically for 4 to 5 h at 37°C to give a reading of ~0.4 on the Dade Microscan turbidity meter (Siemens Healthcare Diagnostics, Tarrytown, NY) before dilution in E-buffer (100 ml of buffered peptone water, 50 μl of Tween 20, 5 g of bovine albumin) to a cell density reading of 0.15 using the Dade Microscan turbidity meter. Cell densities 0.40 and 0.15 correspond to approximately 9.1×10^8 CFU/ml and 3.0×10^8 CFU/ml, respectively. A 450-μl aliquot of each dilution was adjusted to pH 2, 3, 4, or 5 using 1 N hydrochloric acid. An untreated control was included for each strain. The tubes were mixed and rotated at room temperature for 1 h using a Labnet LabRoller rotator (Edison, NJ). After the 1-h treatment, 50 μl of the suspension was added to 2.5 ml of modified tryptic soy broth (mTSB; pH 7.4) without novobiocin and incubated overnight for ~18 h to determine if the cells were lethally injured at the different pH levels. An optical density reading at ~18 h was recorded using the Dade Microscan to demonstrate growth or lack of growth following the various pH treatments. One experiment was conducted on three consecutive days.

Additionally, to assess cell death due to acid treatment during exponential-phase growth, a single colony from each of the six STEC strains was used to inoculate 3 ml of BHI broth. Inoculated broths were incubated at 37°C for 4 to 5 h to reach exponential growth (~0.50 on the Dade Microscan turbidity meter corresponding to approximately 1.0×10^9 CFU/ml). The BHI culture was then serially diluted 10-fold in E-buffer to 10^3 CFU/ml. From the 10^3 CFU/ml suspension, two 450-μl aliquots were transferred to two sterile microcentrifuge tubes for each serogroup. To one 450-μl aliquot, 25 μl of 1 N HCl was added for acid treatment at approximately pH 2 to 2.5, and the other aliquot served as the untreated control. Both acid-treated and untreated samples were rotated for 1 h on a Labnet LabRoller rotator at room temperature. After 1 h, 475 μl of E-buffer was added to the acid-treated samples and 500 μl to the untreated sample. All samples were mixed by vortexing, and a 100-μl aliquot was plated onto BHI plates and incubated for 18 to 24 h at 37°C. Following incubation, colonies for treated and untreated samples were counted. This experiment was performed in triplicate on one day.

Inoculation strategy for the target STEC recovery experiments. Ground beef matrix of approximately 85% lean–15% fat was obtained from Texas A&M University (College Station). The ground beef was negative for the presence of *E. coli* O157 and the top six STEC using the BAX *E. coli* O157:H7 MP test kit (Dupont Qualicon, Wilmington, DE) and the non-O157 screening method described in USDA MLG chapter 5B.01, respectively *(2).* Additionally, the ground beef matrix was found to contain approximately 1.32 log CFU/g aerobic bacteria using APC Petrifilm (3M, St. Paul, MN). Each target and background organism inoculum was prepared by creating a culture suspension in 0.85% saline corresponding to approximately 10^9 CFU/ml (0.5 on the Dade Microscan turbidity meter). The culture suspension was serially diluted 10-fold to 10^2 CFU/ml. From the estimated 10^2 CFU dilution, 10 APC Petrifilm cards were inoculated to determine actual CFU per milliliter. The inocula were then held at 2 to 8°C overnight prior to inoculating samples. Colony counts from the incubated Petrifilm APC cards were then averaged and the inocula were adjusted with 0.85% saline to give target levels of 5 CFU/ml and 500 CFU/ml for the low and high inocula, respectively. Sample portions (325 g) were then inoculated with a 1-ml volume of the appropriate inoculum. Each sample set also contained an uninoculated control. Further, each sample, regardless of inoculum level, was inoculated with the six confounding organisms listed in Table 1 at 2,000 CFU each to give 12,000 CFU per sample. Levels of each confounding organism were chosen to be approximately 3 log higher than target to simulate high gram-negative background microflora compared with low STEC contamination levels. Following inoculation, sample sets along with controls were immediately enriched with 975 ml of mTSB (Acumedia, Neogen Corp., Lansing, MI) with 8 mg/liter sodium novobiocin as described in USDA MLG chapter 5B.01 *(2)* and incubated statically for 15 h at 42°C. For each serogroup, 20 samples were inoculated with four strains (five samples each) with a target level of 5 CFU/325-g sample, and five samples were inoculated with a target level of 500 CFU/325-g sample. At the same time as sample inoculation, a 1-ml aliquot of the refrigerated inoculum was inoculated onto an APC Petrifilm card for each sample inoculated. Petrifilm cards (25 cards per serogroup) were incubated at 37°C, and colonies were counted the following day to give the average CFU level per sample.

Recovery procedure for target STEC from artificially inoculated samples. The IMS procedure was performed as described previously with a few modifications *(2, 11).* Commercially available Dynabeads for serogroups O26, O103, O111, and O145 were purchased from Invitrogen (Carlsbad, CA). IMS beads specific for serogroups O45 and O121 (not commercially available) were received from USDA Agriculture Research Service, Eastern Regional Research Center, Wyndmoor, PA *(11).* Briefly, 1 ml of the 15-h enrichments was filtered through a 40-μm-pore-size filter and mixed with 20 μl of the serogroup-specific IMS beads. Tubes containing enrichment and IMS beads were rotated for 10 min at 20 to 25°C on the Labnet LabRoller rotator. Enrichments with IMS bead reagents were then added to MACS cell separation columns (Miltenyi Biotech, Auburn, CA) and washed 4× with 1 ml of E-buffer prior to elution with 1 ml of E-buffer. Column eluates were diluted 1:10 and 1:100 and 100 μl was spread plated onto RBA and mRBA. Additionally, a post-IMS acid treatment step (1 h at approximately pH 2 to 2.5) was added to help reduce growth of nontarget organisms that adhered to the IMS beads. Briefly, 450 μl from the undiluted, post-IMS column eluate was transferred to a sterile microcentrifuge tube and 25 μl of 1.0 N HCl was added to the tube. The tubes were rotated on the Labnet LabRoller rotator for 1 h at 20 to 25°C. After the 1-h incubation, 475 μl of E-buffer was added to the tube to increase the pH (5.0 to 5.5). A 100-μl aliquot from the tube was then spread plated onto mRBA. Additionally, each enrichment was filtered and directly streaked without IMS onto mRBA using a sterile 10-μl loop. All plates were incubated for 18 to 24 h at 37°C. Following incubation, colonies from the RBA, mRBA, and acid-treated mRBA plates were tested by latex agglutination using latex beads coated with

TABLE 2. *Effect of pH levels on exponential phase of cell growth*

Organism	Optical density reading[a]				
	Untreated	pH 5	pH 4	pH 3	pH 2
Morganella morganii	1.09 ± 0.11	1.12 ± 0.06	1.12 ± 0.09	1.11 ± 0.06	1.14 ± 0.04
Enterobacter cloacae	0.95 ± 0.02	0.93 ± 0.04	0.93 ± 0.07	0.30 ± 0.47	0.02 ± 0.02
Citrobacter freundii	1.09 ± 0.03	1.08 ± 0.05	1.09 ± 0.04	0.04 ± 0.02	0.04 ± 0.02
Enterobacter sakazakii	0.94 ± 0.03	0.89 ± 0.04	0.91 ± 0.05	0.03 ± 0.01	0.04 ± 0.02
Enterobacter cloacae	0.93 ± 0.02	0.91 ± 0.06	0.93 ± 0.06	0.24 ± 0.38	0.03 ± 0.02
Klebsiella pneumoniae	1.01 ± 0.02	1.07 ± 0.04	1.04 ± 0.03	1.04 ± 0.03	0.03 ± 0.01
Escherichia coli O26:H2	0.98 ± 0.01	1.01 ± 0.02	0.96 ± 0.03	0.99 ± 0.05	0.97 ± 0.04
E. coli O45:H2	0.98 ± 0.02	1.01 ± 0.01	1.03 ± 0.03	1.01 ± 0.01	1.01 ± 0.01
E. coli O103:H2	0.99 ± 0.04	1.01 ± 0.02	1.03 ± 0.01	1.02 ± 0.01	1.02 ± 0.02
E. coli O111:H$^-$	0.97 ± 0.07	0.98 ± 0.01	1.01 ± 0.01	0.99 ± 0.01	1.00 ± 0.02
E. coli O121:H19	1.02 ± 0.02	1.04 ± 0.02	1.02 ± 0.03	1.03 ± 0.02	0.99 ± 0.03
E. coli O145:H$^-$	1.02 ± 0.02	1.02 ± 0.02	1.03 ± 0.04	1.03 ± 0.04	1.02 ± 0.02

[a] Cells were treated at each pH for 1 h at room temperature and then inoculated into mTSB for approximately 18 h of incubation. The average optical density readings ($n = 3$) ± standard deviations from the Dade Microscan turbidity meter are shown.

serogroup-specific antisera obtained from USDA Agriculture Research Service, Eastern Regional Research Center, Wyndmoor, PA. Colonies exhibiting a latex-positive reaction were restreaked onto TSA with 5% sheep blood and incubated at 37°C for 18 to 24 h. From these plates, colonies were again tested by latex agglutination. Latex-positive colonies from the TSA with 5% sheep blood were biochemically identified as *E. coli* using the VITEK and/or VITEK2 and genetically confirmed using the real-time PCR assays described in USDA FSIS MLG chapter 5B.01 *(2).*

Statistical analyses. Statistical analysis of the data obtained from the pure culture acid treatment experiment was performed using two-way analysis of variance with Bonferroni's posttest using GraphPad Prism 5.0 software (San Diego, CA). Statistical analysis of the data obtained from the recovery experiments was performed by chi-square analysis using GraphPad Prism 5.0 software. Fisher's exact test was used for comparing groups of two. For comparing more than two groups, chi-square analysis was used. Significance was defined as having a P value < 0.05.

RESULTS

Effect of acid treatment procedure on viability of cells in pure culture. Cells (both STEC and confounding organisms) were subjected to pH levels ranging from 2 to 5 for 1 h and resuscitated in nonselective media for ~18 h. As shown in Table 2, lethally injured cells showed no growth after incubation in the nonselective mTSB (without novobiocin), whereas the growth of representative STEC strains did not appear to be affected by the various pH levels. Of the confounding organisms tested, *Morganella morganii* was the most acid tolerant during our treatment procedure. As shown in Figure 1, pure culture experiments demonstrated that cell death of the six STEC serotypes tested was not significantly increased following acid treatment.

Recovery of target STEC from 15-h enrichments of artificially inoculated ground beef samples. IMS with serogroup-specific IMS beads was performed on all sample enrichments followed by plating onto mRBA or RBA. Table 3 summarizes results for each serogroup for untreated plating onto RBA or mRBA and acid-treated post-IMS eluates plated onto mRBA. There was a significant difference ($P = 0.0004$) in the recovery of O26 using mRBA and acid treatment versus plating without treatment onto RBA. For serogroup O103, there was a significant increase ($P < 0.0001$) in recovery when samples were plated onto mRBA with or without acid treatment versus plating untreated eluates onto RBA. Samples inoculated with low levels of serogroup O111 had significantly ($P = 0.0013$) higher recovery when treated with acid compared with plating of untreated samples onto either mRBA or RBA. There was no significant difference for recovery of serogroups O45, O121, and O145 on RBA or mRBA with or without acid treatments. There was no overall difference ($P = 0.1192$) in recovery of all serogroups on mRBA with and without acid treatment; however, choosing latex-positive colonies was more easily facilitated due to a reduction in background microflora. Figure 2 illustrates an example of the effectiveness of acid treatment for serogroup

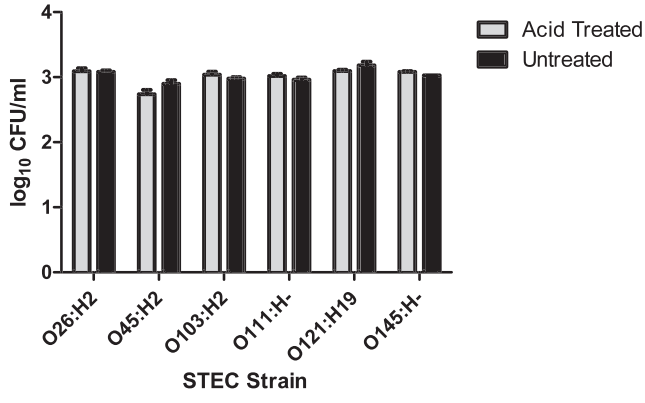

FIGURE 1. *Acid treatment of cells during exponential phase does not cause a reduction in STEC cell numbers. Viable cell counts in acid-treated cell suspensions for each strain were compared with untreated cell suspensions and plated onto BHI agar for viable cell counts. There were no significant differences between treatments for each strain. Standard error of the mean is shown for each strain and treatment.*

TABLE 3. *Recovery of STEC from low-level inoculated ground beef samples using IMS with or without acid treatment*

Serogroup	Inoculation level (CFU/325-g sample)[a]	RBA untreated	mRBA untreated	Acid[b]	*P* value
O26	4.4 (2.7–6.2)	8/20[c]	15/20	19/20	0.0004
O45	3.8 (2.9–4.8)	20/20	19/20	18/20	0.3490
O103	3.2 (2.5–3.9)	4/20	14/20	19/20	<0.0001
O111	3.2 (2.4–3.9)	0/20	0/20	6/20	0.0013
O121	4.5 (3.3–5.6)	20/20	20/20	20/20	NA[d]
O145	3.9 (3.1–4.6)	16/20	20/20	18/20	0.1118
Total	NA	68/120	88/120	100/120	<0.0001

[a] The average inoculum of target STEC into each sample is listed along with the 95% confidence interval.
[b] Post-IMS eluates that received acid treatment were diluted 1:2 and 100 μl was plated onto mRBA.
[c] Number of samples with at least one confirmed positive colony/number of samples inoculated.
[d] NA, not applicable. Chi-square analysis cannot be performed on data sets with 100% recovery for all groups.

TABLE 4. *Recovery of STEC from high level–inoculated ground beef samples using direct streaking of the enrichment without IMS or plating of post-IMS eluates with and without acid treatment*[a]

Serogroup	RBA untreated	mRBA untreated	Acid[b]	DS[c]	*P* value
O26	5/5[d]	5/5	5/5	5/5	NA[e]
O45	5/5	5/5	5/5	5/5	NA
O103	5/5	5/5	5/5	5/5	NA
O111	0/5	0/5	5/5	1/5	0.001
O121	5/5	5/5	5/5	5/5	NA
O145	3/5	4/5	5/5	5/5	0.229
Total	23/30	24/30	30/30	26/30	0.048

[a] Ground beef samples (325 g) were inoculated with 500 CFU.
[b] Post-IMS eluates that received acid treatment were diluted 1:2 and 100 μl was plated onto mRBA.
[c] Colonies recovered by direct streaking (DS) were tested with latex agglutination reagents but did not undergo a confirmation procedure.
[d] Number of samples with at least one confirmed positive colony/number of samples inoculated.
[e] NA, not applicable. Chi-square analysis cannot be performed on data sets with 100% recovery for all groups.

O121. In this figure, it is clear that the acid treatment procedure is effective at reducing the growth of nontarget bacteria that bind the IMS beads and are subsequently plated onto the agar. The reduction of nontarget bacteria facilitates the selection of morphologically correct colonies for latex agglutination or other downstream confirmatory tests such as PCR or biochemical identification.

In Table 4, comparisons are shown for the recoveries of target STEC serotypes from samples inoculated at a level of 500 CFU/325-g sample using RBA (untreated 1:10 and 1:100 dilutions), mRBA (untreated 1:10 and 1:100 dilutions), acid treatment followed by plating onto mRBA, or direct streaking from enrichments. The recovery of serogroup O111 was significantly higher ($P < 0.0001$) for the acid-treated eluates plated onto mRBA compared with plating of the untreated eluates onto RBA or mRBA. Interestingly, we were able to recover target STEC serotypes in 26 (86.7%) of 30 of the high-inoculated (500 CFU/325 g) samples when using the direct streaking method without IMS. Enrichments of samples inoculated with low CFU (1 to 5) also were streaked for isolation without IMS. The number of inoculated samples ($n = 20$) per serogroup in which latex-positive colonies were recovered is as follows (number of samples with latex-positive colonies shown in parentheses): O26 (4), O45 (6), O103 (4), O111 (0), O121 (6), and O145

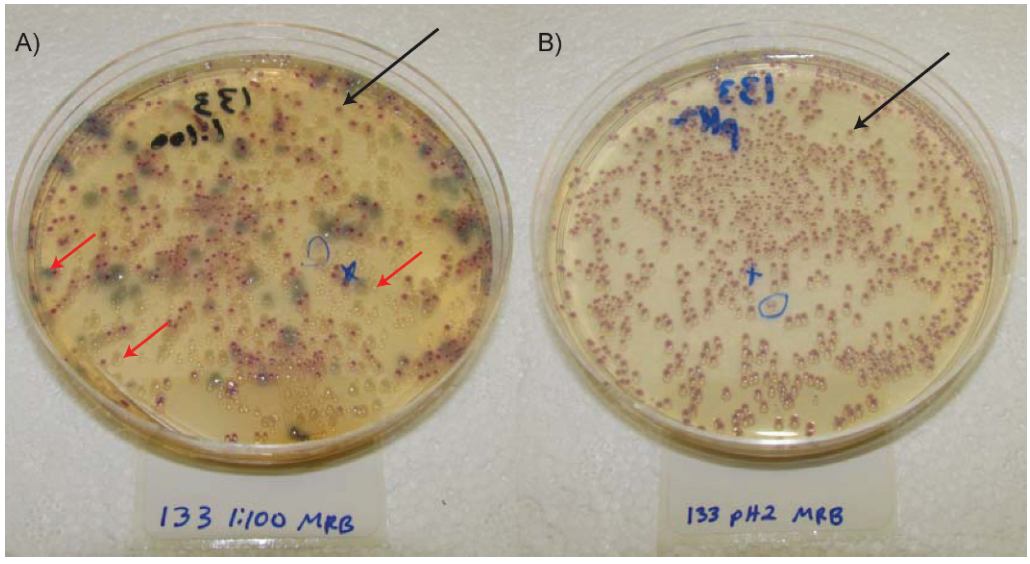

FIGURE 2. *Example of the effectiveness of acid treatment on recovery of* E. coli *O121:H19 on (A) mRBA with no acid treatment and (B) mRBA following acid treatment. The pink colonies identified by the long black arrow in panels A and B are indicative of the target O121:H19 strain. The confounding organisms are indicated by the short red arrows in panel A. Note that a 1:100 dilution of post-IMS eluate was plated in panel A, whereas the acid-treated post-IMS eluate in panel B was diluted 1:2 prior to plating.*

(14). No further confirmation (i.e., PCR or biochemical identification) was performed on latex-positive colonies.

DISCUSSION

In this study, we report an isolation procedure for the top six non-O157 STEC using mRBA and an additional post-IMS acid treatment procedure. Modifying the antimicrobial supplements in the plating medium was necessary due to reports that the potassium tellurite level (0.8 mg/liter) found in RBA inhibits the growth of some STEC strains *(12)*. There is a need to reduce strain selection bias by lowering antimicrobial supplements that affect target STEC growth; however, this must be balanced with the ability to recover target STEC from a complex microfloral background that grows when antimicrobial supplement concentrations are decreased. Our preliminary work showed that the lower tellurite level (0.15 mg/liter) found in mRBA would allow growth of strains previously inhibited by RBA, including two strains from serogroup O103 (serotypes O103:H2 and O103:H11). In addition to the strains from serogroup O103, our preliminary work showed that the lower potassium tellurite level (0.15 mg/liter) allowed growth of strains from serogroups O45, O111, and O121, which were inhibited on agar containing a higher tellurite concentration (0.8 mg/liter). The growth inhibition of some strains from serogroup O111 on TC-SMAC has been reported previously *(12)*. Recently, the growth inhibition of some strains from serogroups O103, O121, and O145 was reported due to inclusion of potassium tellurite at 2.5 mg/liter, while growth was supported by agars lacking potassium tellurite *(16)*. However, in our preliminary work, strain O45 (strain RM2048) did not grow on agar containing potassium tellurite at 0.15 mg/liter or 0.80 mg/liter, indicating that even the lower level of potassium tellurite inhibited growth of this strain.

In conjunction with the use of mRBA, an acid treatment procedure was optimized to take advantage of the acid tolerance of STEC while reducing the growth of other confounding organisms *(7, 12, 13)*. Our findings agree with previous reports that document the acid sensitivity of non–*E. coli* organisms such as *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp., whereas the *Morganella* strain appeared to be acid tolerant using our acid stress procedure *(13)*. While the acid tolerance of every organism associated with raw ground beef could not feasibly be tested with our procedure, the organisms tested are robust organisms that were isolated from enrichment media with high levels of sodium novobiocin (20 mg/liter) and RBA plates containing high levels of potassium tellurite (0.8 mg/liter) and sodium novobiocin (10 mg/liter). The STEC strains tested in this study tolerated the acid treatment procedure, incurring no lethal injury to the cells, as observed with the confounding organisms tested (for example, *E. aerogenes, K. pneumoniae*).

We demonstrated that using a combination of mRBA and acid treatment would increase the likelihood of isolation of the top six non-O157 STEC strains. We were able to isolate and confirm target STEC in 103 (85.8%) of 120 of the low-inoculated samples (1 to 5 CFU/325-g sample) by plating both acid-treated and untreated samples onto mRBA, compared with 68 (56.7%) of 120 of low-inoculated samples without acid treatment and plating onto RBA. In the recovery experiments, we found that two of the four serogroup O103 strains did not grow on RBA although they did grow on mRBA. Again, this is potentially due to the sensitivity of some O103 strains to potassium tellurite found in the plating medium *(12, 16)*. However, recovery of strains from serogroup O111 was problematic on both mRBA and RBA, with no recovery from samples inoculated with a low level of target. Recovery was noted only on 30% of the low-inoculum samples plated onto mRBA following acid treatment. The potential of the acid treatment to reduce background microflora seems especially important for serogroup O111 due to the potential nonspecificity of anti-O111 IMS beads. In every sample inoculated with O111 strains, we could detect O111 by PCR (data not shown), but recovery from the plating media was low. This could be a combination of reduced growth levels in the primary enrichments due to competition of the added confounding organisms and could be due to nonspecific binding of IMS beads with background microflora, reducing binding sites for the target O111 strains. High background microflora with morphologies of the confounding organisms inoculated into the ground beef were noted on the plating media (even on the blank control sample, inoculated with no target but only with 12,000 CFU of background flora) following IMS treatment.

Of note, we were able to recover STEC strains from 26 of 30 (high inoculum, 500 CFU/325-g sample) of the inoculated ground beef samples using direct streaking without an IMS procedure. This could potentially be important for outbreak situations in which there are no IMS reagents available for a serogroup. Acid treatment and direct streaking of the enrichment could potentially provide a quick, inexpensive way to isolate STEC of serogroups outside of the top six serogroups without using IMS reagents; however, this was not experimentally tested in this study and would require further evaluation. Overall, the combination of IMS, acid treatment, and mRBA used in this study was effective for isolation of STEC serogroups O26, O45, O103, O111, O121, and O145 from raw ground beef.

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